Potential of confocal microscopes to resolve in the 50–100 nm range

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We determine the resolution of high-performance confocal microscopes by measuring the three-dimensional point–spread function (3D-PSF) of an optimized confocal setup. The 3D-PSF is standardized by recording the scattered light of pointlike objects. For a wavelength of 543 nm and a specified numerical aperture of 1.4 (oil), we find an axial and lateral focal full width at half-maximum (FWHM) of 460±20 and 145±10 nm, respectively. A high signal-to-noise ratio is obtained by using recording times comparable to those of near-field scanning optical microscopy. We further reduce the effective PSF extent by means of a three-dimensional deconvolution technique exploiting the information gained from the measurement of the focus. We show that it is possible to obtain an axial and lateral FWHM of the far-field effective PSF after deconvolution of 80 and 40 nm, respectively. © 1996 American Institute of Physics. [S0003-6951(96)01750-0]

Scanning optical microscopy is undergoing a rapid growth that is nurtured by the challenge to overcome the classical resolution limits.\(^1\) In the last decade, powerful near-field optical microscopes evolved with lateral resolutions ranging down to the nanometer scale.\(^2\)–\(^5\) In spite of this remarkable achievement, far-field microscopes remained attractive, primarily because of their potential in three-dimensional (3D) imaging. Far-field microscopes are able to obtain information from the interior of a translucent specimen, such as cells or cell nuclei. Thus, near-field optics cannot remain the only approach in the quest for optical super-resolution. It is important to also develop far-field microscopes with resolution well beyond the diffraction limit, and indeed, such microscopes have been proposed.\(^6\)–\(^10\)

Recent activities indicate that this area is growing rapidly, both for near- and far-field approaches. Hence, it becomes of importance to establish the ultimate performance of classical far-field techniques and reveal their potentials and limitations.

It is common practice to compare the resolution of novel near- and far-field microscopes with the confocal microscope because the resolution of a confocal microscope is improved by a factor of 1.4 over its conventional counterpart.\(^11\),\(^12\) In addition, the confocal microscope is the most popular technique for 3D imaging.\(^13\) However, it is important to realize that for historical reasons most of the (commercially available) confocal microscopes are not optimized for highest possible imaging quality or signal, but for more practical factors, such as image acquisition speed and ruggedness. Fast imaging is accomplished by scanning the focused beam with typical dwell times of 1–100 \(\mu\)s. Due to the limited off-axis correction of the lenses and other technical compromises, image fidelity as well as signal-to-noise ratio (SNR) is traded off against speed. The imaging capabilities of a confocal microscope can be improved by scanning the object rather than the beam as it has been realized in the pioneering work by Sheppard, Wilson, and Brakenhoff in the late 1970s.\(^11\)–\(^14\) Brakenhoff et al. were the first to determine high aperture confocal resolution in fine measurements, but these measurements were for holes in a thin metal sheet and could not entirely reveal 3D-point resolution.\(^14\) The introduction of modern 3D-piezoelectric scanners in stage scanning microscopy by Hell and co-workers\(^15\),\(^16\) allowed a considerable improvement of stage scanning, so that precision point–spread function (3D-PSF) measurements and 3D imaging became possible for the highest numerical apertures.

We determine the 3D-PSF of an optimized confocal microscope by scanning randomly dispersed point objects at low speed. We optimized the optical performance of the confocal microscope by using fixed beams that are parallel to the optical axis. With parallel beams and infinity corrected optics we were able to reach the predicted resolution limit. We also aimed at a high (SNR) by imaging scattering gold beads. The high SNR allows for a determination of the PSF. It is known that with a high SNR and a detailed knowledge of the 3D-PSF, a considerable resolution improvement by mathematical methods can be achieved in fluorescence microscopy.\(^18\)–\(^22\) Image formation with scattered light is different from fluorescence imaging but for pointlike objects the PSF measured with scattered light is equivalent to that of fluorescence.\(^11\)–\(^13\) We carried out our experiments with two intentions. First, we aimed at the determination of the confocal 3D resolution under scanning conditions similar to those in near-field optical microscopy. This should result in a standard for resolution in far-field imaging. Second, we applied mathematical techniques to explore the extent to which the 3D-PSF can be reduced and to assess a further potential gain in resolution.

Figure 1 sketches the optical arrangement. The light source is a HeNe laser operating at a wavelength of 543 nm. The light passed an illumination pinhole and was expanded by a lens to ensure a planar illumination of the entrance pupil. The objective lens was a Plan Apochromat (Leica...
100x) with a specified numerical aperture of 1.4 (oil), which is the highest aperture at present. The detection path was symmetric to the illumination path, featuring a pinhole in front of a blue sensitive photomultiplier. The illumination and detection pinholes had a diameter of 10 μm, which was about three times smaller than the back-projected Airy disk, corresponding to about one optical unit.

The specimen consisted of 50 nm gold beads mounted in immersion oil (~n = 1.518). The beads were randomly dispersed in the embedding medium. A drop of the suspension was placed between two standard cover glasses. The object was scanned by a 3D-piezoelectric stage (Melles Griot, Cambridge, England). Generally, we recorded 3D stacks consisting of 30 XY images that were 40 nm apart in the Z direction. The pixel size was 10 nm in the X and Y direction. Figure 2(a) shows a rendered plot of the bead images. The axial elongation of the PSF is readily observed. As the size of the beads is less than one-seventh of the wavelength in the medium, we can assume that the bead images represent the experimental confocal PSF and, therefore, the confocal 3D resolution.

Figure 3 depicts the profiles of one of the measured confocal PSFs along with their theoretical counterparts. The theoretical curves were calculated with a high aperture scalar theory. Taking into account measurements by Wilson and Juskaitis, we chose an aperture of 1.35 rather than the 1.4 specified. The experimental lateral and axial full width at half-maximum (FWHM) are determined to 145±10 and 460±20 nm, respectively. This is in good agreement with the theoretical values of 149 and 420 nm, for the lateral and axial FWHM. In optical units, the experimental lateral and axial resolution is 2.35 and 6.87, respectively. Thus, the lateral and axial resolutions are 1/3.7 and 1/1.2 of the used wavelength, respectively.

In fluorescence microscopy, the precise knowledge of the 3D-PSF allows for an increase in resolution by deconvolution. Our recordings were of scattered light and not fluorescence, but the beads were fairly separated from each other and the light scattered from different beads could hardly interfere, so that we could apply deconvolution. The quality of the measurement and the variance of the PSF in the image enabled us to assess how narrow the effective PSF can be made for the above SNR and scanning fidelity. To deconvolve the images, we used a procedure to establish the 3D-PSF as described in (Ref. 17) and the maximum likelihood estimation (MLE) algorithm. We selected the MLE algorithm because it is optimally suited to restore photon limited images. We selected one of the 3D-PSFs of the stack and deconvolved the stack in Fig. 2(a). The result is shown in Fig. 2(b). The corresponding lateral and axial profiles are shown in Figs. 3(a) and 3(b) in comparison to the raw data. The extent of the PSF is decreased by a factor of 3.6 in lateral direction and 5.75 in axial direction. The axial and lateral FWHM were 80 and 40 nm, respectively. This amounts to an axial resolution of 1.2 and a lateral resolution of 0.64 in optical units. The axial and lateral FWHM of the PSF is about 6.8 and 13.6 times smaller than the wavelength, respectively.

The above data reveal the potential of optimized far-field microscopy for imaging in the 50–100 nm resolution range. What are the limitations of this technique? The most prominent limitations of the restoration approach are finite SNR ratio conditions, the variance of the PSF in the object, and the fact that the MLE image restoration is object dependent: pointlike objects can be restored more effectively and with less sensitivity to noise than larger objects. For the cells the resolution increase will be less efficient. However, in many cases, the fluorescence label is concentrated on a small area, e.g., for specific DNA labels, so that a considerable resolution increase is expected with the method described herein.

We have shown that an improved scanning fidelity can...
be achieved by optimizing a confocal setup for image quality rather than for speed. The use of scattered light allowed us to raise the SNR in our test experiments. This cannot be done in fluorescence imaging without reaching limits that are imposed by the photochemistry of the fluorophore. High quantum efficiency detectors, such as avalanche photodiodes can replace fast but less sensitive photomultipliers in the red regime. The maximum SNR is determined by the collection efficiency of 5%, one is able to gather 5 × 10^3 photons. To exclude bleaching, one could refrain to maximum signal of 5 × 10^5 photons. With a sampling of 16 volume elements per focal volume, one is able to achieve a signal of about 3 × 10^2 photons per volume element. Indeed, we have first evidences that with high efficiency detection and increased recording time lateral FWHM values of 60–70 nm are obtainable for pointlike fluorescence objects. Another limitation is the variance of the PSF in the specimen. Refractive mismatch-induced aberrations were avoided. This was not the case in regular specimens in the past. However, the new water immersion lenses can alleviate this problem. The confocal fluorescence microscope is not the far-field microscope with the highest resolution. 4Pi-confocal microscopy can provide a focus that is sharpened up by physical methods. The doubled aperture is able to provide twice the fluorescence signal per object volume. An axial resolution of 140 nm for a two-photon excitation image at a wavelength of 750 nm has been demonstrated. Deconvolution of 4Pi-confocal images will further decrease the axial FWHM. Besides, concepts for overcoming the lateral resolution limit by physical methods have been proposed, for example offset beam overlap,6,9 STED,7 and GSD fluorescence microscopy.8 Once realized at sufficient SNR, the PSFs of these microscopes can further be enhanced by deconvolution.

We have shown that with an optimized confocal microscope one is able to reach a lateral resolution of nearly one-fourth of the wavelength. Furthermore, we have shown that with a good SNR and scanning fidelity the resolution can be mathematically improved by another factor of 3–4. One can conclude, that with far-field microscopy one should be able to achieve resolutions surpassing a wavelength by more than one order of magnitude.

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